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Electrostatic Interactions of S4 Voltage Sensor in Shaker K^+ Channel

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Summary

The S4 segment comprises part of the voltage sensor in Shaker K^+ channels. We have used a strategy similar to intragenic suppression, but without a genetic selection, to identify electrostatic interactions of the S4 segment that may be important in the mechanism of voltage-dependent activation. The S4 neutralization mutations K374Q and R377Q block maturation of the protein, suggesting that they prevent proper folding. K374Q is specifically and efficiently rescued by the second site mutations E293Q and D316N, located in putative transmembrane segments S2 and S3, respectively. These results suggest that K374, E293, and D316 form a network of strong, local, electrostatic interactions that stabilize the structure of the channel. Some other double mutant combinations result in inefficient suppression, identifying weak, presumably long-range electrostatic interactions. A simple structural hypothesis is proposed to account for the effects of the rescued double mutant combinations on the relative stabilities of open and closed channel conformations.

Introduction

Voltage-dependent ion channels control action potential propagation and initiate neurotransmitter release and therefore are central to information transfer and synaptic function in neurons. Because many voltage-dependent K^+, Na^+, and Ca^{2+} channels share a similar structural organization (Jan and Jan, 1989), it is often presumed that they activate by a common mechanism (Sigworth, 1993). Despite the development of detailed kinetic models describing voltage-dependent gating (Sigworth, 1993; Bezanilla et al., 1994; Zagotta et al., 1994), the physical mechanism of activation has not been described in terms of channel structure.

During voltage-dependent activation, charged amino acids in the protein sense a change in the transmembrane voltage and initiate conformational changes that open the channel. These charged residues contribute to the gating charge of the channel, and their movement can be detected as gating currents (Armstrong and Bezanilla, 1973).

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Two challenges in understanding the physical mechanism of voltage-dependent activation are identifying residues in the channel protein that contribute to the gating charge and describing the conformational changes that transfer it.

We are studying the mechanism of activation in Shaker K^+ channels. Shaker channels are highly voltage dependent, opening in response to depolarization of the membrane potential. The equivalent of 12–14 electronic charges are estimated to be transferred across the transmembrane electric field during the activation of a single Shaker channel (Schoppa et al., 1992; S. K. Aggarwal and R. MacKinnon, personal communication). In a tetrameric channel (MacKinnon, 1991), this would correspond to about 3 charges transferred per subunit. This large transfer of charge implies that a number of charged residues are partially or completely buried in the transmembrane domain of the protein because surface charges would not interact as effectively with the transmembrane electric field.

The S4 sequence, a putative transmembrane segment that is conserved among many voltage-dependent K^+, Na^+, and Ca^{2+} channels (Jan and Jan, 1989), contains a number of positively charged amino acids and comprises at least part of the voltage sensor (Stuhmer et al., 1989; Papazian et al., 1991; Liman et al., 1991; Logothetis et al., 1992). Evidence has been presented suggesting that a basic amino acid, R368, in the Shaker S4 sequence may contribute significantly to the gating charge (Perozo et al., 1994). In Shaker channels, the gating charge on the voltage sensor moves in at least two phases (Schoppa et al., 1992; Bezanilla et al., 1994; Zagotta et al., 1994). The neutralization mutation R368Q greatly decreases the valence of the second component of charge movement, q_2, and the proportion of the total charge it carries (Perozo et al., 1994; Bezanilla et al., 1994). These results suggest that R368 responds to changes in the transmembrane voltage, undergoing a conformational change during the q_2 phase of the activation mechanism. In contrast, residues that contribute to the first component of charge movement, q_1, have not been identified.

Mutational analysis indicates that individual S4 residues make different contributions to the mechanism of activation (Papazian et al., 1991; Perozo et al., 1994). The results rule out simple models of activation involving equivalent S4 charges moving through a uniform electric field. To explain their different contributions to activation, we have proposed that basic residues at different S4 positions experience different environments or structural interactions that determine their roles in voltage-dependent gating (Perozo et al., 1994).

The roles of the S4 residues K374 and R377 in activation have not been studied in detail, because the neutralization mutations K374Q and R377Q result in proteins that fail to mature properly, thereby eliminating channel function (Perozo et al., 1994). One possibility is that these mutations disrupt electrostatic interactions with acidic residues, such as buried or partially buried salt bridges, that are

essential for proper folding or assembly. It has been proposed that S4 residues are stabilized by forming ion pairs with acidic residues (Catterall, 1986; Durrell and Guy, 1992), but no evidence for such interactions has been presented. Any acidic amino acid that is located in the membrane domain or that interacts with S4 basic residues may contribute to the gating charge of the channel and the mechanism of activation.

To test the hypothesis that K374Q and R377Q disrupt electrostatic interactions with transmembrane acidic residues, we have used a strategy identical in concept to intragenic suppression but without a genetic selection. Intragenic suppression of folding defects is a well-established genetic approach to identify interacting regions in a protein (Chen et al., 1991; Fane and King, 1991; Portillo et al., 1991; King et al., 1991; Lee et al., 1992; Meunier et al., 1993). Using this approach, two ion pairs between charged amino acids in transmembrane segments have been identified in the lactose permease, a membrane transport protein of Escherichia coli (King et al., 1991; Lee et al., 1992; Sahin-Toth and Kaback, 1993). Neutralization of one charged partner in a pair knocks out transport function. A second site suppressor, identified using genetic selection, specifically neutralizes the oppositely charged partner, restoring function (King et al., 1991; Lee et al., 1992; Sahin-Toth et al., 1992; Dunten et al., 1993; Sahin-Toth and Kaback, 1993). These results provide the rationale for our strategy. If K374Q and R377Q disrupt electrostatic interactions, it should be possible to suppress the defects in maturation and function by neutralizing particular acidic amino acids.

We have obtained both strong and weak suppression of the primary mutations K374Q and R377Q, identifying likely short- and long-range electrostatic interactions among transmembrane charged residues. The functional effects of the rescued double mutant combinations indicate that the interactions occur in the final structure, not just in folding intermediates. A simple structural model is proposed to account for some of the functional effects of the double mutant combinations.

Results

Strong and Weak Intragenic Suppression of K374Q and R377Q

Shaker protein made in Xenopus oocytes is detected on SDS gels as two glycosylated bands (Santacruz-Toloza et al., 1994). One is a sharp band of about 70 kDa, whereas the second, which represents most of the protein, is a broad band of about 110 kDa (Santacruz-Toloza et al., 1994; see Figure 2). A similar pattern of Shaker expression is seen in transfected HEK293T cells, in which pulse-chase experiments have shown that the lower molecular weight band is an immature, partially glycosylated precursor that is converted to the diffuse mature form of the protein by the attachment of additional carbohydrate (Schulteis et al., 1995). These results suggest that the immature form represents core-glycosylated protein from the endoplasmic reticulum, whereas the mature band has been further processed in the Golgi apparatus and includes cell surface protein (Rowling and Freedman, 1993). K374Q and R377Q make only the partially glycosylated, putative endoplasmic reticulum form of the protein (Perozo et al., 1994; see Figure 2). Because misfolded membrane proteins are retained in the endoplasmic reticulum, these results suggest that K374Q and R377Q fail to fold properly (Gething et al., 1986; Copeland et al., 1988).

To test the hypothesis that K374Q and R377Q disrupt essential electrostatic interactions, we paired these S4 mutations with neutralization mutations of acidic amino acids predicted by hydrophobicity analysis and topology models to be in membrane spanning segments (Tempel et al., 1987; Miller, 1991). The acidic positions E283 and E293 in putative transmembrane segment S2, D316 in S3, and D447 in the pore-forming region (P region), which are highly conserved among different voltage-dependent K^+ channels (Chandy and Gutman, 1994), were included in our analysis (Figure 1). D431 in the P region was not included because compelling evidence has been presented that its charged side chain is exposed in the external mouth of the pore (MacKinnon and Yellen, 1990; MacKinnon et al., 1990). In some structural models, the side chain of D447 also faces the pore (Durrell and Guy, 1992; Bogusz et al., 1992), but no definitive evidence has been reported.

The S4 mutation K374Q was paired individually with the acidic neutralization mutations E283Q, E293Q, D316N, and D447N in a construct that contained a deletion of amino acids 6–46 to remove N-type inactivation (Hoshi et al., 1990). This inactivation-removed (IR) background is

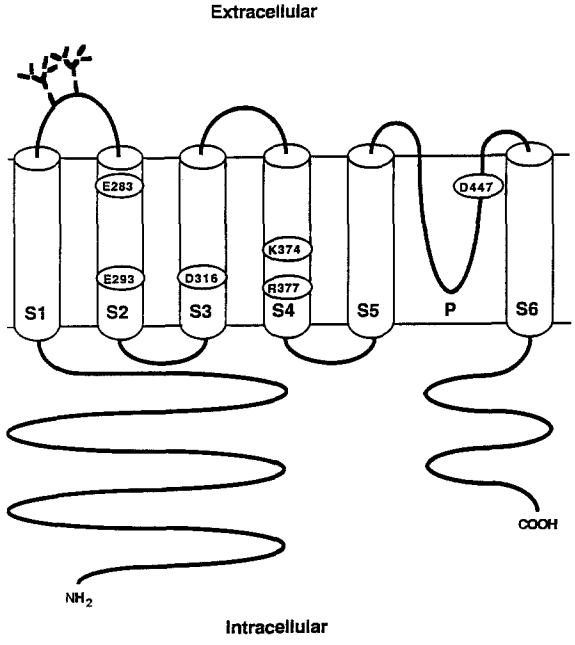

Figure 1. A Model for the Topology of a Shaker Subunit in the Membrane

The putative transmembrane segments S1–S6 and the P region are indicated. The approximate positions of the acidic and basic residues included in our experiments are labeled. Also shown are two sites (N259 and N263) in the S1–S2 loop that are modified by N-linked glycosylation (Santacruz-Toloza et al., 1994).

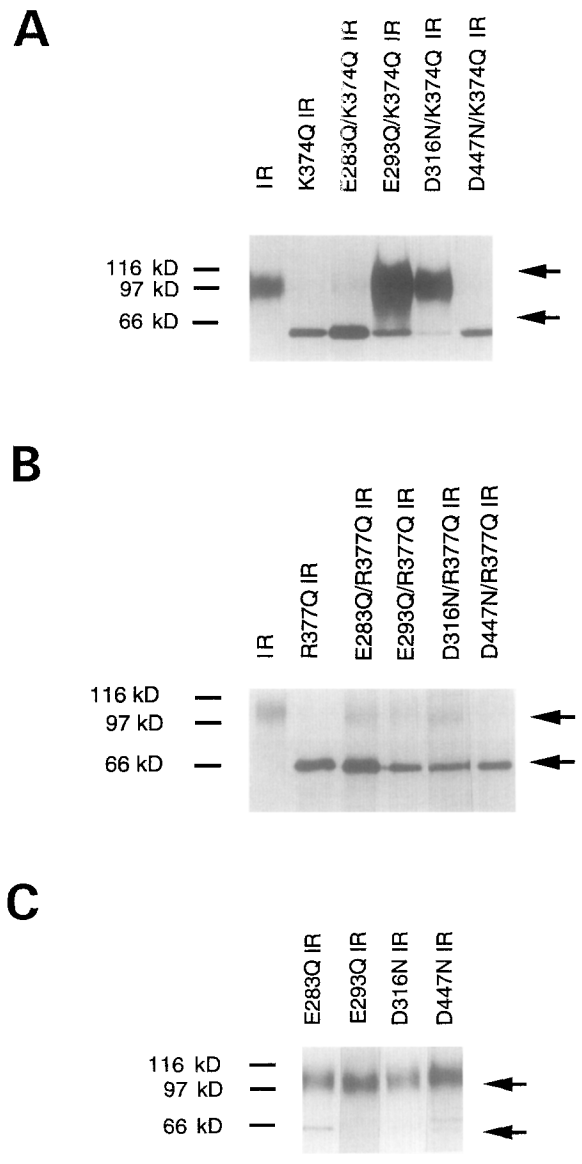

Figure 2. Intragenic Suppression of the Maturation Defects in K374Q and R377Q

(A) Suppression of the maturation defect of K374Q-IR in double mutant combinations. K374Q-IR was paired individually with E283Q, E293Q, D316N, and D447N. After expression and metabolic labeling in Xenopus oocytes, the protein products of Shaker-IR, K374Q-IR, and the double mutant combinations were isolated by immunoprecipitation and subjected to electrophoresis and autoradiography. The arrows mark the positions of the mature (upper) and immature (lower) forms of the protein.

(B) Suppression of the maturation defect of R377Q-IR in double mutant combinations. R377Q-IR was paired individually with E283Q, E293Q, D316N, and D447N and subjected to biochemical analysis along with Shaker-IR and R377Q-IR. The mature Shaker-IR protein is shown at roughly the same intensity as the mature protein produced by the double mutant combinations of R377Q-IR with E283Q, E293Q, and D316N to demonstrate that these inefficiently rescued combinations make proportionately much more immature than mature protein compared with Shaker-IR.

(C) Protein products of the acidic neutralization mutations E283Q-IR, E293Q-IR, D316N-IR, and D447N-IR. The band running just above the immature band in the D447N-IR lane is occasionally seen in constructs that mature normally. It appears to be due to an as yet unidentified posttranslational modification of the Shaker protein that is correlated with maturation. The fraction of the protein in the mature form was estimated by densitometry: Shaker-IR, 99%; K374Q-IR, 0%; K374Q-IR + E283Q, 5%; K374Q-IR + E293Q, 89%; K374Q-IR + D316N, 97%; K374Q-IR + D447N, 0%; R377Q-IR, 0%; R377Q-IR + E283Q, 8%; R377Q-IR + E293Q, 13%; R377Q-IR + D316N, 23%; R377Q-IR + D447N, 0%; E283Q-IR, 93%; E293Q-IR, 100%; D316N-IR, 99%; D447N-IR, 99%.

advantageous for electrophysiological analysis and seems to increase the level of protein production. The double mutant combinations were expressed in Xenopus oocytes, and their protein products were compared with those of Shaker-IR and K374Q-IR 44–48 hr after injection of RNA (Figure 2A). As previously reported, K374Q-IR makes only the immature form of the protein (Perozo et al., 1994). Two double mutant combinations, K374Q-IR + E293Q and K374Q-IR + D316N, suppressed the maturation defect of K374Q efficiently, generating significant amounts of mature protein. One combination, K374Q-IR + E283Q, rescued the defect inefficiently, restoring only a small amount of the protein to the mature form. In contrast, the combination K374Q-IR + D447N failed to rescue the maturation defect; no mature protein was detected (Figure 2A).

After pairing R377Q (in the IR background) with the same acidic neutralization mutations, different results were obtained (Figure 2B). Three of the double mutant combinations, R377Q-IR + E283Q, R377Q-IR + E293Q, and R377Q-IR + D316N, inefficiently rescued the maturation defect of R377Q-IR. Similar to K374Q-IR + E283Q, these combinations produced proportionately small levels of the mature protein. In contrast, the double mutant combination R377Q-IR + D447N did not suppress the maturation defect.

Individually, each of the acidic neutralization mutations made both mature and immature Shaker proteins in proportions similar to the IR control (Figure 2C). Furthermore, E283Q-IR, E293Q-IR, and D316N-IR expressed functional channels (data not shown). Although D447N-IR produced a normal protein pattern, it generated no detectable ionic currents after expression in oocytes (data not shown). D447N-IR may be similar to another mutation in the P region, W434F. W434F eliminates the ionic current but apparently produces mature cell surface protein because it has normal gating currents (Perozo et al., 1993). Whether D447N-IR has normal or altered gating currents is unknown.

If the double mutants restore proper folding and structure, channel function may also be restored. Channel activity was assayed using a two electrode voltage clamp. Production of the mature form of the protein was correlated with the generation of functional channels (Figure 3). The efficient rescue of K374Q-IR maturation by either E293Q or D316N was accompanied by high levels of channel activity. The constructs K374Q-IR + E283Q, R377Q-IR + E293Q, and R377Q-IR + D316N, which partially rescued the maturation defect, produced smaller currents (Figure 3). Only R377Q-IR + E283Q, which resulted in proportionately more of the immature protein than either R377Q-IR

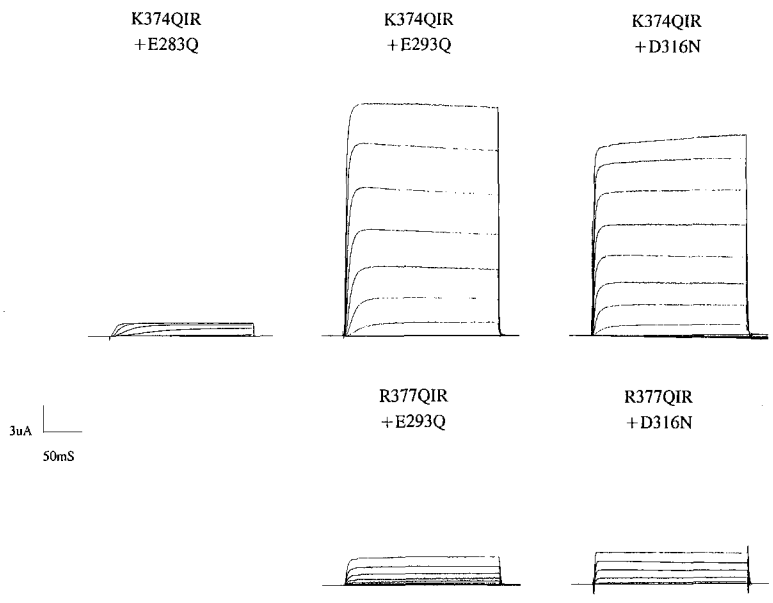

Figure 3. Functional Rescue of K374Q-IR and R377Q-IR in Double Mutant Combinations

Double mutant combinations were analyzed for functional activity in Xenopus oocytes using a two electrode voltage clamp. Comparable amounts of RNA were injected for each construct, and current amplitudes were determined 2–3 days later. Currents were recorded at 14°C ± 1°C in modified Barth's saline (Timpe et al., 1988) after depolarizations from a holding potential of −80 mV to test potentials ranging from −60 to +80 mV in 20 mV increments. Representative traces are shown. The mean current amplitudes ± SEM at +80 mV were as follows: K374Q-IR + E283Q, 1.6 ± 0.07 μA (n = 5); K374Q-IR + E293Q, 18.9 ± 10.6 μA (n = 2); K374Q-IR + D316N, 17.9 ± 5.2 μA (n = 4); R377Q-IR + E293Q, 2.7 ± 0.8 μA (n = 5); R377Q-IR + D316N, 4.2 ± 0.7 μA (n = 11). R377Q-IR + E283Q did not express detectable currents (data not shown).

+ E293Q or R377Q-IR + D316N (see Figure 2B), failed to produce detectable levels of ionic current. From these results, we conclude that the level of current in the double mutant combinations reflects the amount of the mature form of the protein, and that the mature protein folds properly into the functional structure. K374Q-IR + D447N and R377Q-IR + D447N, which did not generate any detectable mature protein, did not express any detectable current (data not shown). However, because D447N-IR made mature protein in amounts similar to IR (see Figure 2C) but produced no ionic currents, the failure to restore maturation is a more reliable indication that D447N does not suppress the defects of K374Q.

Characterization of the Voltage-Dependent Properties of the Double Mutant Combinations

Because K374Q and R377Q are inactive, little is known about the contribution of K374 and R377 to the activation of Shaker channels. Therefore, the voltage-dependent properties of K374Q-IR or R377Q-IR paired with E293Q or D316N were characterized using macropatch methods and compared with those of Shaker-IR and the individual acidic neutralizations E283Q-IR, E293Q-IR, and D316N-IR (Figure 4; Table 1). K374Q-IR + E283Q did not express high enough levels of current for macropatch analysis. Many of the single and double neutralization mutants reduced the steepness of the activation curve (Figure 4; Table 1), suggesting that the charged amino acids normally present at these positions may contribute to the gating charge of the channel. Mutants that shifted activation in the depolarized direction (right) reduced the slope more dramatically than those that shifted activation in the hyperpolarized direction (left). Those mutants with a shallow, right-shifted conductance–voltage (g–V) curve resemble R368Q, an S4 mutation that reduces the valence of the second component of gating charge movement (Perozo et al., 1994). Because a shallow, right-shifted g–V curve could result from changes in the activation mechanism other than a reduction in the gating valence of the channel (Schoppa et al., 1992; Perozo et al., 1994), gating current experiments will be necessary to determine whether these single and double mutants alter the gating charge of the channel.

The position of the g–V curve in the double mutants was determined by the presence of the S4 mutations K374Q or R377Q, not by the acidic neutralizations (Figure 4). K374Q-IR + E293Q and K374Q-IR + D316N shifted activation in the hyperpolarized direction, whereas R377Q-IR + E293Q and R377Q-IR + D316N shifted activation in the depolarized direction. In contrast, E293Q-IR alone shifted activation to the left, whereas D316N-IR alone shifted activation to the right. These functional effects suggest that electrostatic interactions between the S4 basic residues and the acidic amino acids in S2 and S3 occur in the final structure, not just in folding intermediates.

Discussion

Suppression Identifies Likely Short- and Long-Range Electrostatic Interactions among Buried, Charged Residues

After pairing K374Q or R377Q with E283Q, E293Q, or D316N, two levels of suppression were observed. Efficient rescue was accompanied by large amounts of mature protein and large currents, whereas partial rescue was accompanied by inefficient restoration of maturation and small or no currents (see Figure 2 and Figure 3). These results are consistent with the idea that K374Q and R377Q disrupt electrostatic interactions with acidic residues in putative transmembrane segments S2 and S3, which are important for proper folding of the protein. In contrast, K374 and R377 do not appear to interact electrostatically with D447 in the P region. We propose that efficient rescue identifies strong electrostatic interactions, whereas poor rescue identifies weak interactions.

Because electrostatic interactions can extend over fairly

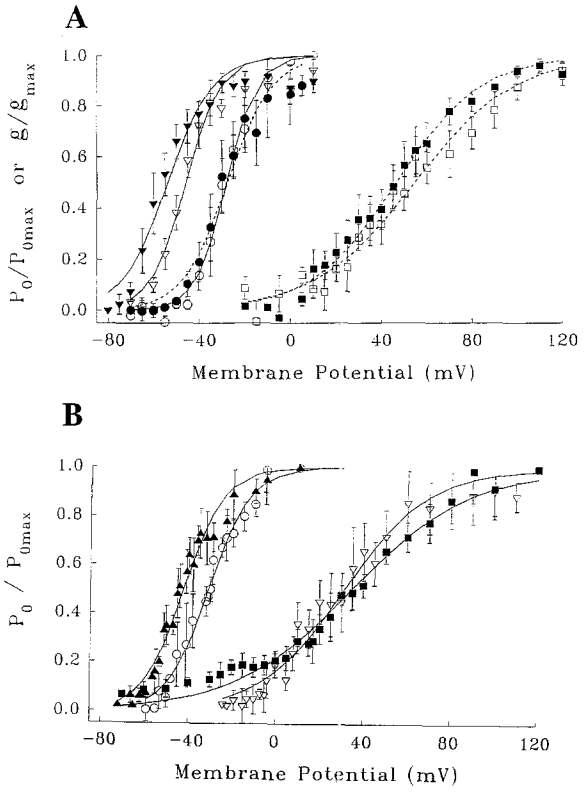

Figure 4. Characterization of the Voltage Dependence of Single and Double Mutants

Conductance–voltage (g–V) curves of (A) Shaker-IR (open and closed circles) and double mutant combinations K374Q-IR + E293Q (open triangles), K374Q-IR + D316N (closed triangles), R377Q-IR + E293Q (open squares), and R377Q-IR + D316N (closed squares) and (B) Shaker-IR (open circles) and acidic neutralization mutants E283Q-IR (open triangles), E293Q-IR (closed triangles), and D316N-IR (closed squares). The voltage dependence of activation was characterized using macropatch methods. For high expressing constructs, the open probability (P_o) was determined as a function of voltage from isochronal tail currents recorded at −80 mV (solid curves). For partial rescue constructs that expressed at a lower level, a normalized, steady-state g–V curve was derived from current amplitudes at the indicated potentials (dashed curves). Activation of the Shaker-IR control was analyzed using both methods with similar results. The data (± SEM, n as in Table 1) were fit with a Boltzmann equation (solid or dashed curves).

Table 1. Activation Parameters for Double and Single Mutants

Construct	V_{mid} (mV)	Slope Factor (mV)	n	Shift
Shaker-IR (isochronal tail method)	−28 ± 3.2	5.6 ± 0.2	4	—
Shaker-IR* (steady-state g–V method)	−27 ± 4.6	8.4 ± 2.5	4	—
K374Q-IR + E293Q	−45 ± 2.9	7.6 ± 1.2	6	Left
K374Q-IR + D316N	−52 ± 4.6	11 ± 1.8	5	Left
R377Q-IR + E293Q*	+55 ± 6.8	21 ± 2.0	4	Right
R377Q-IR + D316N*	+46 ± 6.4	16 ± 0.6	6	Right
E283Q-IR	+50 ± 8.8	19 ± 3.5	8	Right
E293Q-IR	−45 ± 2.4	9.3 ± 1.4	6	Left
D316N-IR	+40 ± 4.9	28 ± 3.2	16	Right

The data in Figure 4, obtained from 4 to 16 different patches, were fit with a Boltzmann equation of the form

$$P_o(V) = P_{o,max}/(1 + \exp[(V_{mid} - V)/A])$$

to obtain values for V_{mid} and the slope factor (A), shown ± SEM. $P_o/P_{o,max}$ or g/g_{max} values were obtained either from isochronal tail current measurements at −80 mV (no symbol) or from steady-state ionic currents (indicated by asterisk). Results for Shaker-IR obtained using both methods are shown. The direction of the shift of activation compared with the IR control is indicated.

long distances (10–20 Å) in a low dielectric environment (Russell et al., 1987; Gilson and Honig, 1988), K374Q and R377Q may disrupt both short- and long-range interactions. We suggest that the more complete the rescue, the closer the positions are during folding or in the final structure. Because strong, short-range interactions make much larger contributions to protein stability than weak, long-range electrostatic interactions, neutralizing nearby acidic residues restores proper folding and function much more effectively than neutralizing more distant residues. Therefore, our results suggest that K374 interacts electrostatically at a relatively short range with E293 and D316, whereas R377 interacts with E293 and D316 over a longer distance. In addition, both K374 and R377 interact with E283 over a long range. Rescue by E283Q was the least efficient, consistent with the topological model for the Shaker subunit in which position 283 is further from 374 and 377 than are 293 and 316 (see Figure 1).

Electrostatic interactions among ionized residues that are at least partially buried in the transmembrane domain provide the best explanation for our data. First, the observation of weak suppression is consistent with the idea that the interactions disrupted by K374Q and R377Q are electrostatic in nature. We interpret weak suppression to indicate fairly long-range interactions. Electrostatic interactions extend over fairly long distances in proteins, but their strength diminishes with distance (Matthew, 1985; Honig et al., 1986; Gilson and Honig, 1988). (The dielectric constant of the protein between two charges, which is unknown, will also affect the strength of the electrostatic interaction between them [Gilson and Honig, 1986].) No other types of structural interactions that stabilize proteins extend beyond a few angstroms. Second, the severity of the original mutations suggests that the residues are buried and charged. If K374 and R377 were surface residues, the mutations K374Q and R377Q would not be expected to disrupt protein folding so severely. In addition, if K374 and R377 were unprotonated (i.e., neutral), replacement with glutamine, which has a similar volume (Chothia, 1984), might be easily tolerated. Finally, allosteric effects due to the substitution of residues with different space-filling properties cannot account for the suppression. The original residues and the substituted glutamines have similar mean volumes when buried in proteins (Chothia, 1984). For instance, the residues lysine plus glutamate fill a volume of 326 $Å^3$ when buried in a protein, whereas two glutamines fill a volume of 322 $Å^3$ (Chothia, 1984).

If the original electrostatic interaction was so important for folding, why does a second neutralization mutation suppress the defects of the first? Neutralizing one charged

residue in a buried ion pair makes it energetically infeasible to fold the oppositely charged partner into its proper location in the structure. Neutralizing the partner removes this barrier. Other structural interactions are apparently then strong enough to fold the protein into the functional structure.

We have assumed that K374Q and R377Q disrupt folding of the channel subunit. In this case, the electrostatic interactions would stabilize the tertiary structure of the channel. However, the results do not preclude the possibility that the mutations instead disrupt subunit assembly. In this case, the electrostatic interactions identified between K374 and R377 and acidic residues in S2 and S3 would be between subunits, stabilizing the quaternary structure of the channel. However, because charged interactions among transmembrane segments may form before being inserted into the bilayer (Honig and Hubbell, 1984), a priori, it seems more likely that the mutations disrupt folding rather than assembly.

K374 May Form a Charge Network with E293 and D316

Because both E293Q and D316N rescue K374Q efficiently, K374 appears not to be stabilized primarily by a one-to-one salt bridge or ion pair with a particular amino acid. Instead, K374, E293, and D316 may be part of a network of charged interactions similar to those found in the interior of globular proteins (Barlow and Thornton, 1983; Rashin and Honig, 1984). In such a network, the charged residues would presumably be stabilized by additional interactions, including hydrogen bonds (Honig and Hubbell, 1984). In soluble proteins and in the lactose permease, charged residues in networks and ion pairs are separated by 4 Å or less (Barlow and Thornton, 1983; Rashin and Honig, 1984; Jung et al., 1993). However, our results address only the relative proximity of residues, not their absolute physical separation.

A network of one basic and two acidic amino acids could explain why the neutralization K374Q disrupts protein folding and structure more severely than either E293Q or D316N. Either of the acidic residues can be neutralized without disrupting the functional structure, presumably because the balanced charge pair that remains does not create an energetic barrier to folding. Neutralizing K374 is fatal for protein folding and function, however, because two negative charges would be left in close proximity in the structure.

Because R377Q was not rescued efficiently in these experiments, we conclude that R377 interacts most closely with as yet unidentified amino acids.

A Structural Model to Account for the Effects of the Double Mutants on the Relative Stability of Open and Closed Conformations

Double mutant combinations containing K374Q shifted activation in the hyperpolarized direction, whereas double mutant combinations containing R377Q shifted activation in the depolarized direction. The position of the g–V curve reflects the relative stability of the closed and open conformations of the channel protein. The hyperpolarized shifts

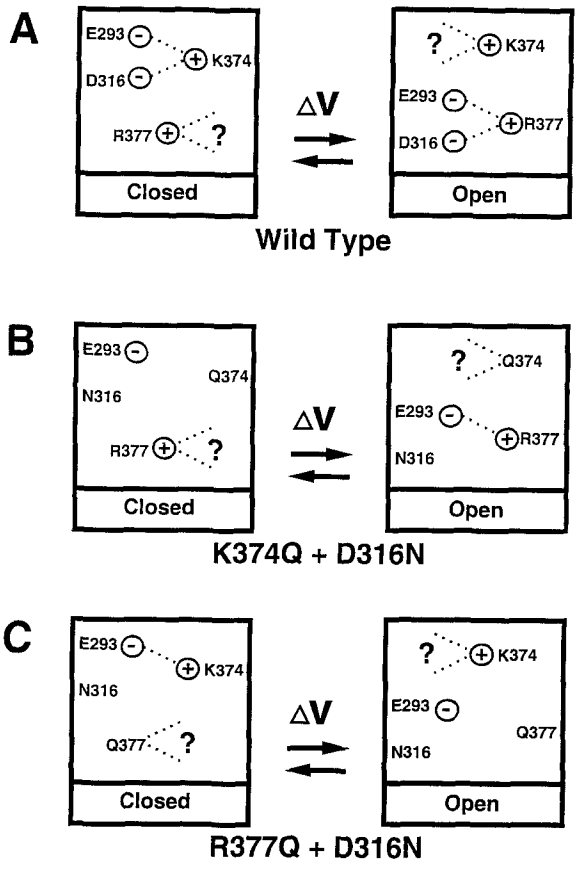

Figure 5. A Model of Electrostatic Interactions to Account for the Shifts in Activation Caused by the Double Mutant Combinations

(A) Wild-type channel. In the closed state (left), E293 and D316 interact at short range with K374, whereas R377 is stabilized by interactions with as yet unidentified amino acids. After the channel opens (right), E293 and D316 interact at short range with R377, whereas K374 is stabilized by unidentified interactions.

(B) K374Q + D316N. The model has been redrawn with neutralizations at positions 316 and 374. In this double mutant, the closed state has an unpaired acidic residue, whereas in the open state, E293 and R377 form a balanced charge pair. This charge difference would be expected to decrease the stability of the closed state relative to the open state, shifting the g–V curve in the hyperpolarized direction.

(C) R377Q + D316N. The model has been redrawn with neutralizations at 316 and 377. In this double mutant, E293 is paired with K374 in the closed channel but is unpaired in the open channel. This charge difference would be expected to decrease the relative stability of the open state, shifting the g–V curve in the depolarized direction.

of K374Q-IR + E293Q and K374Q-IR + D316N indicate that they increase the stability of the open state relative to closed states. In contrast, R377Q-IR + E293Q and R377Q-IR + D316N decrease the stability of the open state relative to closed states. A simple model of structural interactions can be proposed to account for the effects of the double mutants on the relative stabilities of the open and closed conformations (Figure 5). According to this hypothesis, E293 and D316 interact at short range with K374 in the closed, wild-type channel, whereas R377 is stabilized by interactions with as yet unidentified amino acids (Figure 5A). After the channel opens, E293 and D316 interact at short range with R377, whereas K374 is stabilized by un-

identified interactions. In the wild-type channel, there is an extra negative charge near K374 in the closed channel and near R377 in the open channel. These extra charges would not be expected to have a large effect on the relative stabilities of open and closed conformations because both states have an extra charge. In the double mutant K374Q + D316N, E293 is unpaired in the closed state, whereas E293 and R377 form a balanced charge pair in the open state (Figure 5B). This charge difference between the two conformations would be expected to decrease the stability of the closed state relative to the open state, shifting the g–V curve in the hyperpolarized direction. An analogous argument can be made for K374Q + E293Q. In the double mutant R377Q + D316N, E293 interacts with K374 in the closed channel but is unpaired in the open (Figure 5C). This charge imbalance would be expected to increase the relative stability of the closed state, shifting the g–V curve in the depolarized direction. An analogous argument can be made for R377Q + E293Q.

This hypothesis takes into account the proposed transmembrane topology of the Shaker subunit (Miller, 1991) and the direction of electrostatic forces that would act on acidic and basic residues when the membrane is depolarized. It is an oversimplification on at least two counts, however. First, biophysical analysis indicates that Shaker channels have several closed states (Zagotta and Aldrich, 1990; Bezanilla et al., 1991), whereas the model considers only one closed conformation. Second, the model is not meant to show all of the interactions these residues experience in different protein conformations, nor to suggest that unpaired charges or charge pairs are buried in the protein in the absence of additional stabilizing interactions, such as hydrogen bonds (Honig and Hubbell, 1984; He and Quiocho, 1993).

This hypothesis suggests that the conformation important for folding in the endoplasmic reticulum resembles the closed conformation, because this is the conformation in which K374 is proposed to interact at short range with D316 and E293. In addition, the model suggests that the relative positions of S2, S3, and S4 change during activation, consistent with the requirement for buried charges to undergo voltage-dependent conformational changes. Liman et al. (1991) have previously proposed that every other basic S4 residue interacts with an acidic amino acid in an ion pair when the channel is closed, whereas the alternate basic residues form ion pairs when the channel is open. The acidic partners were not identified, however.

In summary, suppression of the maturation and functional defects of K374Q and R377Q by second site mutations in transmembrane acidic residues has provided experimental evidence for electrostatic and structural interactions among segments S2, S3, and S4. The results suggest that K374, R377, E283, E293, and D316 are charged and located in the transmembrane domain, where they are candidates to contribute to the gating charge of the channel. The strategy of directed intragenic suppression can be used to identify other likely, short-range structural interactions, leading to a model for the packing of transmembrane segments. This approach has the potential to provide a bridge between two dimensional topology models and a model for the tertiary packing of transmembrane segments.

Experimental Procedures

Molecular Biology

Mutations were generated (Papazian et al., 1991; Santacruz-Toloza et al., 1994) in a Bluescript subclone of the Shaker B cDNA (Schwarz et al., 1988), verified by dideoxy sequencing, and where indicated, combined by transferring appropriate restriction fragments. RNA was prepared and injected into Xenopus oocytes as previously described (Timpe et al., 1988).

Biochemistry

For biochemical analysis, RNA was coinjected with 400 nCi per oocyte of in vitro translation grade [^{35}S]methionine (Santacruz-Toloza et al., 1994). Oocytes (n = 40–60) were harvested at 44–48 hr after injection. Shaker protein was solubilized and immunoprecipitated from a membrane fraction and then subjected to electrophoresis and autoradiography as previously described (Santacruz-Toloza et al., 1994).

Electrophysiology

Two electrode voltage clamp analysis was performed as previously described (Papazian et al., 1991; Santacruz-Toloza et al., 1994; Schulteis et al., 1995).

The voltage dependence of activation was determined from patches with macroscopic currents. Patch pipettes, coated with Sylgard, had resistances of 0.6–1.2 MΩ. The capacitive transient was compensated using the capacitance compensation circuit on the Axopatch-1D amplifier. Uncompensated capacitance and leak currents were subtracted digitally using the P/−4 protocol (Bezanilla and Armstrong, 1977). To measure P_o as a function of voltage from isochronal tail current measurements, the membrane potential was depolarized from −80 mV to the test potential and then repolarized to −80 mV. Tail current amplitudes were determined at 0.6 or 1 ms after repolarization. These amplitudes were normalized to the maximal isochronal current amplitude for that patch and plotted as a function of test potential. The pipette solution for tail current measurements contained 50 mM RbCl, 65 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES (pH 7.2) (Liman et al., 1991). To obtain a steady-state g–V curve, the membrane potential was depolarized from −80 mV to the test potential, and the steady-state current amplitude was determined by averaging over a 15 ms interval. The reversal potential was determined for each patch. The pipette solution contained 98 mM KCl, 14.5 mM NaCl, 1.8 mM $CaCl_2$, and 10 mM HEPES (pH 7.2). Under these conditions, the reversal potential ranged from 0 to −10 mV. The data were normalized to the maximal conductance for each patch. For all patch-clamp experiments, the bath solution contained 98 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES (pH 7.2). For right-shifted mutants, V_{mid} and the slope factor determined by steady-state measurements may be inaccurate, because at the largest depolarizations the channel may have been blocked by internal magnesium (Slesinger et al., 1993), and endogenous currents may have been activated.

The data were fit using a single, first-power Boltzmann equation (see Table 1). This equation implies a two state model of activation, which is an oversimplification for Shaker channels (Zagotta and Aldrich, 1990; Bezanilla et al., 1991). However, using a fourth-power Boltzmann, as suggested by a kinetic model for the channel (Zagotta et al., 1994), resulted in qualitatively similar results as using a first-power Boltzmann; that is, constructs that shifted the g–V curve significantly to the left or right or that reduced its steepness did so no matter how the data were fit (data not shown). Thus, the qualitative effects of the mutations on the apparent voltage dependence of activation and the relative stabilities of the open and closed states do not depend on the assumptions made in fitting the data.

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